Use of Immunotoxins in Combination to Inhibit Clonogenic Growth of Human Breast Carcinoma Cells


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ABSTRACT

Substantial heterogeneity has been observed in the expression of individual antigens within tumor cell populations. Immunotoxins which bind to different cell surface antigens might exert additive or synergistic cytotoxicity when used in combination to eliminate all clonogenic cells within a tumor. Immunotoxins have been prepared by conjugating recombinantly derived toxin A chain to different monoclonal reagents which recognize cell surface determinants of M, 42,000 (317G5), 55,000 (260F9), and 200,000 (741F8). Each immunotoxin was evaluated for binding, internalization, and cytotoxicity with four breast cancer cell lines. Each of the three immunotoxins bound to the SKBr3 cell line and exerted antitumor activity in a limiting dilution clonogenic assay. Simultaneous treatment with two immunotoxins produced additive antitumor activity with each of the possible combinations. Additive binding could be demonstrated by immunofluorescent techniques, however, with only one of three combinations. With two of the three combinations, subpopulations of tumor cells could be identified which lacked one or the other antigenic determinant but not both. Consequently, log-additive antitumor activity was produced by immunotoxins in combination, and heterogeneity of antigenic targets may have contributed to the combined cytotoxicity in some but not all cases.

INTRODUCTION

Immunotoxins which contain monoclonal antibodies linked to toxic polypeptides provide novel and potentially specific reagents to inhibit tumor cell growth (1–5). The antitumor activity of several immunotoxins, including those constructed with ricin A chain, depends upon (a) binding of the conjugate to target antigens, (b) internalization, and (c) translocation of the toxic moiety into the cytoplasm where ribosomes are inactivated catalytically (6). Binding of an immunotoxin to target cells is directly related to the activity of the antibody and to the density of antigenic determinants expressed on the cell surface. Although each malignant tumor cell is thought to arise from a single progenitor, heterogeneity is observed between cells within the same tumor. To the extent that a subpopulation of tumor cells lacks a given antigen, individual immunotoxins may not eliminate target cells quantitatively. To compensate for heterogeneity in antigen expression, different immunotoxins can be targeted to antigenically distinct determinants. Assuming that different antigenic determinants are expressed independently, use of different immunotoxins in combination could produce additive or synergistic inhibitory tumor growth. Use of multiple immunotoxins could also increase the total number of immunotoxin molecules bound per cell.

A number of immunotoxins have been prepared which react with epithelial carcinomas. Recently, Bjorn et al. (7) described the development of 85 different monoclonal antibodies that bind to human breast cancers. Many of the immunotoxins prepared by conjugating these antibodies to ricin toxin A chain were cytotoxic for tumor cells at low concentrations (<1 μM). A substantial fraction of these antibodies, however, did not form potent immunotoxins. From among the most active reagents, three immunotoxins have been prepared which bind to proteins of molecular weight 42,000, 55,000, and 200,000. Each of these reagents binds to a majority of breast cancers, but substantial heterogeneity has been observed in their expression within and between different tumors (8). If heterogeneity of antigen expression actually limits the antitumor activity of any single immunotoxin, a combination of different reagents should produce additive or synergistic antitumor activity against clonogenic breast cancer cells. Moreover, it should be possible to demonstrate additive binding of different immunotoxins or the presence of tumor subpopulations which lack one or the other antigenic determinant.

MATERIALS AND METHODS

Chemicals. Iodogen was purchased from Pierce Chemical Co. (Rockford, IL) and the N-hydroxysuccinimide ester of biotin was obtained from Calbiochem (La Jolla, CA). For labeling antibodies, FITC was purchased from Research Organics, Inc. (Cleveland, OH), avidin-Texas red was bought from Cooper Biomedical Inc. (Malvern, PA), and goat anti-mouse RPE conjugate was purchased from Tago, Inc. (Burlingame, CA). Proteinase K was purchased from Sigma Chemical Company (St. Louis, MO) and Na125I was obtained from Amersham Corp. (Arlington Heights, IL).

Immunotoxins. The murine monoclonal antibodies 317G5, 260F9, and 741F8 are IgGl immunoglobulins which react with determinants of M, 42,000, 55,000, and 200,000 (9), respectively. Each of these determinants is expressed by a majority of human breast cancers (8–11). MOPC21 is a murine IgGl immunoglobulin that does not react with human breast cancer cells. Immunotoxins were prepared by conjugation of rRTA with 2-iminothiolane (12). They were subsequently purified by passage over trisacryl M blue and by gel filtration on ACA44. Immunotoxin preparations were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using gradients of 5–12.5% polyacrylamide. Fractions were quantitated by densitometry. Each immunotoxin contained <3.3% free IgG, 36–39% 1-mer, 30–34% 2-mer, 16–17% 3-mer, and 8–12% highermers. Impurities were <6%.

Cell Lines. Four human breast cancer cell lines were used in this study, BT20 (13), CAMA-1 (14), SKBr3 (15), and BT483 (16). A human foreskin fibroblast line (HUFF) was obtained from Dr. Kay Singer (Duke University Medical Center). The BT20, SKBr3, and BT483 cell lines were maintained in RPMI 1640 medium supplemented with 15% or 5% (BT483) FBS and 0.03% or 0.06% (BT20) glutamine. Medium for BT20 cells was also supplemented with 100 units/ml...
penicillin, 100 μg/ml streptomycin, and 20 μg/ml insulin. CAMA-1 cells were grown in Dulbecco's minimal essential medium supplemented with 15% FBS and 0.03% glutamine. All tissue culture materials were obtained from Hazelton Biologies (Kansas City, MO). All cells were cultured at 37°C in an atmosphere of 5% CO₂ and 95% humidified air. Cultures were split once a week at a ratio of 1:5.

Conjugation of Antibodies with FITC and Biotin. The method of Feteau (17) was followed for coupling of antibody and FITC. For biotinylation of antibody, 1.7 mg of the N-hydroxysuccinimide ester of biotin was dissolved in 1.0 ml of 0.1 N-dimethylformamide, and 200 μl of this solution were added to 10 mg of antibody in sodium bicarbonate buffer (0.1 M, pH 9.0). After incubation for a period of 4 h at room temperature, the reaction mixture was dialyzed extensively against PBS (pH 7.4) at 4°C to remove unreacted biotin. Following conjugation, materials were centrifuged at 40,000 rpm for 20 min to remove aggregates.

Immunofluorescence Assays. Human breast cancer cells (5 × 10⁶) were incubated in microtube flasks (1.5 ml) with antibody-FITC conjugates (50 μl of a 20 μg/ml solution) or antibody-biotin conjugate (50 μl of a 1:20 dilution) for 30 min on ice and were washed 2 times with PBS containing 1.0% FBS. For the binding studies with FITC-antibody conjugates, the cells were fixed with 2% formaldehyde after incubation with antibody. In experiments using biotinylated antibodies, cells were incubated with avidin-Texas red conjugate (1:20 dilution in PBS with 1% FBS) for 30 min on ice, washed, and fixed immediately in 2% formaldehyde. In experiments which utilized RPE conjugates, human breast cancer cells (5 × 10⁶) were incubated in microtube flasks with one murine monoclonal antibody for 30 min on ice. The cells were washed twice in PBS with 1% FBS and incubated with a goat anti-mouse RPE conjugate on ice. After two additional washes, FITC- and RPE monoclonal antibody conjugates were added and incubated for 30 additional min on ice. All cells were analyzed in an Epics 753 flow cytometer (Coulter Electronics, Hialeah, FL). Gates were set to include cells which were only stained with FITC conjugates (quadrant 1), only stained with Texas red or RPE conjugates (quadrant 4), both (quadrant 2), or either (quadrant 3).

Radioiodination of Immunotoxins. Antibody-toxin conjugates were labeled with Na¹²⁵I using the iodogen method (18). In brief, 50 μl of phosphate buffer (0.5 M, pH 7.4) were added to a 15- x 75-mm borosilicate tube coated with 10 μg of iodogen. Immunotoxins (50-100 μg in PBS) were added in a volume of 90 μl. Radioiodination was initiated by the addition of 1 mCi of NaI¹²⁵I (10 μl) and the mixtures were incubated for 30 min on ice. The protein-bound iodine was separated by gel filtration on a PD-10 column (Pharmacia, Pleasant Hill, CA) that had been precollibrated with PBS. A sample of 2 μl from each fraction was counted in a Packard gamma counter to measure protein-bound radioactivity. Iodination efficiency was calculated by the following formula:

\[
\text{Iodination efficiency} = \frac{\text{protein bound cpm} \times 100}{\text{total cpm}}
\]

The efficiency of iodination ranged between 50 and 80%. The iodinated conjugates were separated on 6.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19). Gels were dried and autoradiographed to confirm that the appropriate conjugates had, in fact, been labeled.

Binding of Immunotoxins. A live cell radioimmunoassay was used to measure binding of immunotoxins to target cells. Breast cancer cells were grown as monolayers, trypsinized, washed once with culture medium, and seeded at a density of 1 x 10⁶ cells/well into 96-well flat-bottomed Removewell plates (Costar, Cambridge, MA). After overnight incubation at 37°C, the monolayers were washed and incubated for 2 h at 37°C in RPMI 1640 medium with 10% BSA to block nonspecific binding of the radionuclide conjugate. To measure binding of immunotoxins, different amounts of labeled conjugate were added in a volume of 40 μl and incubated on ice for 4 h. Cells were then washed 4 times with ice-cold medium containing 1% BSA. Individual wells were detached and radioactivity was measured.

Internalization of Immunotoxins. Iodinated immunotoxins (1 μg/ml) were allowed to bind to breast cancer cells (5 x 10⁵) at 4°C for 1 h. The unbound immunotoxin was removed by washing with culture medium containing 1% BSA. Cells were then incubated at either 37°C or 4°C for 1 to 6 h. To distinguish internalized immunotoxin from that which had only bound to the tumor cell surface, cells were treated with proteinase K (100 μl of 2.5 mg/ml solution) for 1 h at 37°C with gentle shaking. Cells were washed 3 times with cold medium containing 1% BSA and 0.1% Na₂SO₄. Radioactivity was measured in a Packard gamma counter.

Clonogenic Assay. To investigate the effect of immunotoxins on breast cancer cells, a clonogenic assay was used (19). In brief, target cells (1 x 10⁶ in 1 ml) were treated with different dilutions of immunotoxins (0.01 to 10 μg/ml) for 3 h at 37°C on a rocking platform, in a humidified atmosphere containing 5% CO₂ and 95% air. Following treatment, the cells were washed twice with tissue culture medium and serially diluted 5-fold. From each dilution, a sample of 100 μl was pipetted into 6 wells of a 96-well plate, an additional 100 μl of medium were added to each well, and the cells were incubated for a period of 14 days. The clonogenic growth of surviving tumor cells was evaluated by inverted phase microscopy, scoring the number of wells with at least 1 colony containing more than 30 cells. Estimates of the most probable number of residual clonogenic units were calculated by a modification of the method of Spearman and Karber (20, 21). The number of clonogenic units is calculated as:

\[
\text{units} = \ln (0.1) + \frac{\text{Ln5}}{2} - \frac{\text{Ln5}}{6} \sum r_i
\]

where \(r_i\) = all wells with growth. In all assays, control untreated cultures were used to determine the cloning efficiency.

Isobolographic Analysis. Isobolographic analysis was performed according to the method of Steel and Peckham (22). Isobolographic analysis considers the dose of each drug required to produce a given antitumor effect, e.g., 99% (2 log) of clonogenic tumor cell elimination (23). If the dose-response curve for each individual agent is linear with respect to drug concentration, a concave isobologram indicates true synergy. If the dose-response curves are nonlinear, as was found in the present study, analysis becomes more complex and a concave isobologram may reflect either additive or synergistic activity. The method of Steel and Peckham (22) calculates an "envelope of additivity" between two modes which indicate the theoretical limits of additive effects. Mode I is calculated based on the assumption that the effect of each agent is independent, whereas Mode II is calculated based on the assumption that the two agents interact. In the present study, synergistic interactions would fall below Mode II, outside the envelope of additivity.

RESULTS

Binding of Antibodies and Immunotoxins to Breast Cancer Cells. Binding of three immunotoxins (317G5-rRTA, 260F9-rRTA, and 741F8-rRTA) was evaluated using four different breast cancer cell lines, BT20, CAMA-1, BT483, and SKBr3. Conjugates were radioiodinated and tested for binding to target cells in a live cell radioimmunoassay. Binding of radiolabeled immunotoxins could be blocked with specific antibody. Representative data are presented in Table 1. Data in Fig. 1 show the relative binding of the immunotoxins to each of the breast cancer cell lines. In this assay, the 317G5-rRTA, which recognizes a M, 42,000 antigen, showed the greatest binding to all four target cell lines. The 741F8-rRTA, which recognizes a M, 55,000 antigen, bound primarily to SKBr3, whereas the 260F9-rRTA, which recognizes a M, 55,000 antigen, bound primarily to the SKBr3 cell line and poorly to the other three cell lines. Consequently, SKBr3 was chosen for further studies to evaluate the use of immunotoxins in combination. With this cell line as target, all three conjugates exhibited maximal binding at a concentration of 15 μg/ml (Fig. 1).

Internalization of Immunotoxins. The antitumor activity of ricin A chain conjugates depends critically upon the ability of
Table 1 Specificity of immunotoxin binding to SKBr3

<table>
<thead>
<tr>
<th>Expt. 1</th>
<th>125I-Immunotoxin</th>
<th>Nonlabeled free antibody</th>
<th>cpm/well</th>
<th>SKBr3</th>
<th>HUFF</th>
</tr>
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<tr>
<td>0</td>
<td>260F9 (5 µg/ml)</td>
<td>0</td>
<td>55 ± 17</td>
<td>58 ± 54</td>
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<tr>
<td>260F9-RTA (1 µg/ml)</td>
<td>260F9 (0.05 µg/ml)</td>
<td>9,274 ± 1,574</td>
<td>1,804 ± 840</td>
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<td></td>
</tr>
<tr>
<td>260F9-RTA (1 µg/ml)</td>
<td>260F9 (0.5 µg/ml)</td>
<td>7,225 ± 1,122</td>
<td>1,603 ± 744</td>
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<tr>
<td>260F9-RTA (1 µg/ml)</td>
<td>260F9 (5 µg/ml)</td>
<td>6,822 ± 1,166</td>
<td>1,280 ± 650</td>
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<tr>
<td>260F9-RTA (1 µg/ml)</td>
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<td>2,276 ± 9,682</td>
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<table>
<thead>
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<th>Expt. 2</th>
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<th>118 ± 64</th>
<th>118 ± 14</th>
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<tr>
<td>0</td>
<td>741F8 (5 µg/ml)</td>
<td>0</td>
<td>181 ± 98</td>
<td>410 ± 344</td>
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<tr>
<td>741F8-RTA (1 µg/ml)</td>
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<td>33,375 ± 9,595</td>
<td>6,467 ± 2,944</td>
<td></td>
</tr>
<tr>
<td>741F8-RTA (1 µg/ml)</td>
<td>741F8 (0.5 µg/ml)</td>
<td>31,414 ± 6,894</td>
<td>4,902 ± 910</td>
<td></td>
</tr>
<tr>
<td>741F8-RTA (1 µg/ml)</td>
<td>741F8 (5 µg/ml)</td>
<td>11,621 ± 2,518</td>
<td>4,000 ± 773</td>
<td></td>
</tr>
</tbody>
</table>

Toxin molecules reach the cytoplasmic compartment. One important determinant of toxin trafficking is likely to be the rate at which bound immunotoxin is internalized by endocytosis. Consequently, experiments were carried out to quantitate the amount of conjugate internalized after antigen binding sites had been saturated. Radioiodinated conjugates were incubated with breast cancer cells at 4°C to permit binding and to prevent endocytosis of the immunotoxin. After removing excess conjugate, the cells were warmed to 37°C to initiate internalization. In preliminary studies, several approaches had been compared to differentiate between conjugate that was bound at the cell surface and conjugate that had been internalized. Attempts were made to remove immunotoxin at the cell surface using trypsin, Pronase, and exposure to low pH buffers. Each of these reagents affected the morphological integrity of tumor cells. Treatment with protease K for 1 h, however, removed more than 90% of membrane-bound conjugate without producing significant cell lysis. Radioactivity that remained after protease K treatment was considered to reflect internalized immunotoxin conjugate. Incubation for 4 h at 37°C produced maximal accumulation of intracellular conjugate (data not shown), and these conditions were chosen to compare different immunotoxins.

Fig. 2 depicts the relative amount of proteinase K-resistant radioactivity associated with the four different breast cancer cell lines. Following binding to available sites, cells were incubated at either 4°C or 37°C for a period of 4 h. Greater amounts of each immunotoxin were internalized at 37°C than at 4°C with each of the four cell lines and for all three conjugates tested. Among the different immunotoxins, 317G5-rRTA produced the highest level of binding to the surface of each cell line, as well as the greatest level of proteinase K-resistant incorporation of radioactivity. Evidence for internalization was obtained for the 260F9-rRTA conjugate with the BT20 cell line and for the 741F8-rRTA with the SKBr3 cell line. Uptake of these immunotoxins by the other cell lines was at the limit of detection by this assay.

Effect of Immunotoxins on the Clonogenic Growth of Breast Cancer Cells. The antitumor activity of the different immunotoxins was assessed against the four different cell lines using
long term clonogenic assays. Tumor cells were exposed to conjugates for a period of 3 h at 37°C, and surviving clonogenic units were enumerated by a limiting dilution assay requiring 2 weeks for growth of viable cells in culture. Previous studies from our laboratory have indicated a direct correlation between visual scoring and incorporation of $[^{3}H]$ thymidine by clonogenic cells in this assay (22). A linear correlation was observed between the number of tumor cells plated and the number of clonogenic units measured over several orders of magnitude (Fig. 3).

The effect of different concentrations of specific immunotoxins on the clonogenic growth of breast cancer cells is shown in Fig. 4. With increasing levels of 317G5-rRTA, there was a dose-dependent decrease in the number of clonogenic units for each of the four cell lines. The 317G5-rRTA conjugate completely inhibited the growth of BT483 and CAMA-1 but did not completely inhibit clonogenic growth of SKBr3 and BT20, even at the highest concentration tested (10 μg/ml). The 260F9-rRTA conjugate was effective against BT20, BT483, and SKBr3 cells, whereas it did not inhibit CAMA-1 cells. The 741F8-rRTA was toxic for the SKBr3 and BT483 cell lines but not for BT20 or CAMA-1 cells. Antitumor activity of the immunotoxins could be blocked with specific nonconjugated antibody (Table 2). A nonspecific immunotoxin, MOPC21-rRTA, failed to affect the growth of the different breast cancer cell lines (Fig. 5).

**Fig. 2.** Internalization of immunotoxins. Breast cancer cell lines were incubated with saturating concentrations of immunotoxins. Subsequently, cells were incubated for 4 h at 4°C or at 37°C. Immunotoxins remaining at the cell surface after incubation were removed by treatment with proteinase K. Radioactivity which remained after enzyme digestion was considered to have been internalized. Values reflect the mean amount of immunotoxin bound ± SD for duplicate samples.

**Fig. 3.** Clonogenic growth of different human breast cancer cell lines. Different numbers of breast cancer cells from four lines (BT20, SKBr3, BT483, and CAMA-1) were plated in the clonogenic assay. Clonogenic units were calculated from the number of wells which contained colonies after 14 days of incubation.

Improved Elimination of Target Tumor Cells by Combined Treatment with Two Immunotoxins. Each of the three immunotoxins exerted antitumor activity against SKBr3. Consequently, it was of interest to determine whether additive antitumor activity could be obtained using immunotoxins in combination. SKBr3 cells were exposed to 317G5-rRTA and 260F9-rRTA conjugates at different concentrations. In Fig. 6, clonogenic growth of SKBr3 cells is shown after treatment with different concentrations of 260F9-rRTA (Fig. 6, upper) or 317G5-rRTA (Fig. 6, lower), in the absence or presence of 1 μg/ml levels of the alternative immunotoxin. Even at high concentrations, the individual immunotoxins eliminated no more than 2 log of clonogenic tumor cells. With both immunotoxins some 4 log of tumor cells could be destroyed.

For more precise analysis, the antitumor activity of several different concentrations of 260F9-rRTA and 317G5-rRTA was assayed in a separate experiment and an isobologram was constructed (22, 23). When used individually, 2.1 μg/ml 317G5-rRTA conjugate or 4.6 μg/ml 260F9-rRTA conjugate was required to inhibit clonogenic tumor cell growth by 1.6 log, i.e., 97.5% (Fig. 7). Substantially lower concentrations of each immunotoxin were required when the two reagents were used in combination. The antitumor activity of each immunotoxin was not, however, linear with respect to immunotoxin concentration (data not shown). Consequently, it was necessary to calculate envelopes of additivity, reflecting the concentrations of each reagent required to produce a particular increment of tumor cell killing. Calculation of envelopes of additivity indicated that the isobole for 1.6 log tumor cell kill fell at the extreme limit of additivity, verging on synergy. Using similar analysis, additive effects were observed with 741F8-rRTA and 260F9-rRTA, as well as with 741F8-rRTA and 317G5-rRTA (Figs. 8 and 9).

**Fig. 4.** With increasing levels of 317G5-rRTA, there was a decrease in the number of tumor cells plated and the number of clonogenic units measured over several orders of magnitude (Fig. 3).

For more precise analysis, the antitumor activity of several different concentrations of 260F9-rRTA and 317G5-rRTA was assayed in a separate experiment and an isobologram was constructed (22, 23). When used individually, 2.1 μg/ml 317G5-rRTA conjugate or 4.6 μg/ml 260F9-rRTA conjugate was required to inhibit clonogenic tumor cell growth by 1.6 log, i.e., 97.5% (Fig. 7). Substantially lower concentrations of each immunotoxin were required when the two reagents were used in combination. The antitumor activity of each immunotoxin was not, however, linear with respect to immunotoxin concentration (data not shown). Consequently, it was necessary to calculate envelopes of additivity, reflecting the concentrations of each reagent required to produce a particular increment of tumor cell killing. Calculation of envelopes of additivity indicated that the isobole for 1.6 log tumor cell kill fell at the extreme limit of additivity, verging on synergy. Using similar analysis, additive effects were observed with 741F8-rRTA and 260F9-rRTA, as well as with 741F8-rRTA and 317G5-rRTA (Figs. 8 and 9).

**Fig. 5.** Improved elimination of target tumor cells by combined treatment with two immunotoxins. Each of the three immunotoxins exerted antitumor activity against SKBr3. Consequently, it was of interest to determine whether additive antitumor activity could be obtained using immunotoxins in combination. SKBr3 cells were exposed to 317G5-rRTA and 260F9-rRTA conjugates at different concentrations. In Fig. 6, clonogenic growth of SKBr3 cells is shown after treatment with different concentrations of 260F9-rRTA (Fig. 6, upper) or 317G5-rRTA (Fig. 6, lower), in the absence or presence of 1 μg/ml levels of the alternative immunotoxin. Even at high concentrations, the individual immunotoxins eliminated no more than 2 log of clonogenic tumor cells. With both immunotoxins some 4 log of tumor cells could be destroyed.

For more precise analysis, the antitumor activity of several different concentrations of 260F9-rRTA and 317G5-rRTA was assayed in a separate experiment and an isobologram was constructed (22, 23). When used individually, 2.1 μg/ml 317G5-rRTA conjugate or 4.6 μg/ml 260F9-rRTA conjugate was required to inhibit clonogenic tumor cell growth by 1.6 log, i.e., 97.5% (Fig. 7). Substantially lower concentrations of each immunotoxin were required when the two reagents were used in combination. The antitumor activity of each immunotoxin was not, however, linear with respect to immunotoxin concentration (data not shown). Consequently, it was necessary to calculate envelopes of additivity, reflecting the concentrations of each reagent required to produce a particular increment of tumor cell killing. Calculation of envelopes of additivity indicated that the isobole for 1.6 log tumor cell kill fell at the extreme limit of additivity, verging on synergy. Using similar analysis, additive effects were observed with 741F8-rRTA and 260F9-rRTA, as well as with 741F8-rRTA and 317G5-rRTA (Figs. 8 and 9).

**Fig. 6.** With increasing levels of 317G5-rRTA, there was a decrease in the number of tumor cells plated and the number of clonogenic units measured over several orders of magnitude (Fig. 3). For more precise analysis, the antitumor activity of several different concentrations of 260F9-rRTA and 317G5-rRTA was assayed in a separate experiment and an isobologram was constructed (22, 23). When used individually, 2.1 μg/ml 317G5-rRTA conjugate or 4.6 μg/ml 260F9-rRTA conjugate was required to inhibit clonogenic tumor cell growth by 1.6 log, i.e., 97.5% (Fig. 7). Substantially lower concentrations of each immunotoxin were required when the two reagents were used in combination. The antitumor activity of each immunotoxin was not, however, linear with respect to immunotoxin concentration (data not shown). Consequently, it was necessary to calculate envelopes of additivity, reflecting the concentrations of each reagent required to produce a particular increment of tumor cell killing. Calculation of envelopes of additivity indicated that the isobole for 1.6 log tumor cell kill fell at the extreme limit of additivity, verging on synergy. Using similar analysis, additive effects were observed with 741F8-rRTA and 260F9-rRTA, as well as with 741F8-rRTA and 317G5-rRTA (Figs. 8 and 9).

**Binding of Two Antibodies to Target Cells.** Since 317G5, 260F9, and 741F8 bind to distinct antigens on the surface of tumor cells, it was of interest to study whether incubation with two antibodies in combination would produce additive accumulation of immunoglobulin. The additive binding of radioiodinated immunotoxins was studied initially using a whole-cell radioimmunoassay. When immunotoxins were evaluated individually, SKBr3 cells bound more 317G5-rRTA than 260F9-rRTA. Incubation of cells with both immunotoxins produced additive binding of cell-associated radioactivity (Fig. 10).

For further analysis was undertaken using directly fluoresceinated antibodies. Fig. 11 shows the additive binding of 317G5-FITC and 260F9-FITC, using one-color analysis. Treatment with 260F9-FITC labeled 68.63% of SKBr3 cells with a mean fluorescence intensity of 17.3. 317G5 bound to 94.51% of target cells, producing a higher mean fluorescence intensity of 44.4. Addition of both antibodies increased the total number of positive cells to 98.25% and further increased the mean fluorescence intensity to 71. In studies with 741F8 and 260F9 or 317G5, additive antibody binding was not, however, observed (data not shown).

Even in the absence of additive binding, log-additive antitumor activity of different immunotoxins might result from the elimination of subpopulations lacking one or the other antigen. Consequently, two-color analysis was carried out to detect 317G5*260F9* and 317G5*260F9* subpopulations. 260F9 was biotinylated and incubated with SKBr3 cells. Addition of avidin-Texas red identified cells that expressed the M, 55,000 antigen recognized by 260F9. Simultaneous incubation with 317G5-FITC permitted the identification of cells which expressed the M, 42,000 determinant recognized by 317G5. In
IMMUNOTOXINS IN COMBINATION

Fig. 4. Effect of specific immunotoxins on the clonogenic growth of different human breast cancer cell lines. Breast cancer cells (1 x 10⁶) from four lines (BT20, SKBr3, BT483, and CAMA-1) were treated with different concentrations (0.01-10 µg/ml) of three immunotoxins (317G5-rRTA, 260F9-rRTA, and 741F8-RTA) for 3 h at 37°C. The cells were washed twice with tissue culture medium, serially diluted, and assayed as described in text.

![Immunotoxin Concentration (µg/ml)]

Table 2 Specificity of immunotoxin activity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Free antibody</th>
<th>Immunotoxin</th>
<th>Clonogenic Growth</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No. of positive wells</td>
</tr>
<tr>
<td>SKBr3</td>
<td>0</td>
<td>0</td>
<td>260F9-RTA (1 µg/ml)</td>
<td>40.5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>250F9-RTA (1 µg/ml)</td>
<td>26.0</td>
</tr>
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Fig. 5. Effect of nonspecific immunotoxin on clonogenic growth of different human breast cancer cells. Breast cancer cells (1 x 10⁶) from four lines (BT20, SKBr3, BT493, and CAMA-1) were treated with different concentrations (0.01-10 µg/ml) of control MOPC21-rRTA immunotoxin for 3 h at 37°C. The cells were washed twice with tissue culture medium, serially diluted, and assayed as described in text.

![Immunotoxin Concentration (µg/ml)]

Fig. 12, two-color analysis indicated that 317G5 bound to 97.26% of SKBr3 cells (quadrants 1+2), whereas 260F9 bound to 68.8% of SKBr3 cells (quadrants 2+4). Approximately 67.3% of tumor cells bound both reagents (quadrant 2). Importantly, 29.96% of tumor cells were 317G5+260F9~, and 1.51% 317G5~260F9+, indicating two subpopulations which could be labeled only by a combination of the two immunotoxins. Similar observations have been made with combinations of 741F8 and 317G5 (Fig. 13) but not with 741F8 and 260F9 (Fig. 14).

DISCUSSION

In the present report, the antitumor activity of three immunotoxins has been evaluated, individually and in combination. Binding and internalization of the different immunotoxins varied between the four cell lines studied. 317G5-rRTA, which recognizes a M, 42,000 antigen, bound most avidly to all four breast carcinoma cell lines. The 317G5-rRTA immunotoxin was internalized by each of the cell lines and was the most
effective of the three immunotoxins in eliminating clonogenic growth in all four cell lines. The 741F8-rRTA, which identifies a M, 200,000 antigen, bound primarily to the SKBr3 cell line. Evidence for internalization was equivocal, but modest antitumor activity was obtained against both SKBr3 and BT483 cells. The 260F9-rRTA, which recognizes a M, 55,000 antigen, bound to the SKBr3 and BT483 cell lines. The most convincing evidence for internalization of 260F9-rRTA was obtained with the BT20 cell line, but cytotoxicity was observed with BT20, SKBr3, and BT483.

The correlation between binding and internalization of immunotoxins was not precise. The largest differences between intracellular activity at 4°C and 37°C were observed with 317G5-rRTA with each of the four cell lines. 317G5-rRTA bound most effectively to each of the same cell lines. 260F9-rRTA was internalized but exhibited relatively little binding to BT20. Conversely, relatively little 260F9-rRTA was internalized by SKBr3, but substantial binding was observed. The 741F8-rRTA did bind most effectively to SKBr3, and significant internalization was observed with this cell line. Interestingly, substantially more immunotoxin bound to cells than was internalized. Relatively high concentrations of immunotoxins were required to inhibit clonogenic tumor growth. Internaliza-
IMMUNOTOXINS IN COMBINATION

Fig. 11. Binding of 260F9 (upper) and 317G5 (middle) to SKBr3 cells alone and in combination (lower), measured by direct fluorescence.

Fig. 12. Coincident binding of 260F9 and 317G5 to SKBr3 cells. Quadrant 1+2, 317G5-FITC; quadrant 2+4, 260F9-biotin-avidin-Texas red binding to SKBr3 cells. Percentage of positive cells was calculated from the analysis of 10,000 cells.

Fig. 13. Coincident binding of 317G5 and 741F8 to SKBr3 cells. Quadrant 1+2, 741F8-FITC; quadrant 2+4, 317G5-RPE.

Fig. 14. Coincident binding of 260F9 and 741F8 to SKBr3 cells. Quadrant 1+2, 741F8-FITC; quadrant 2+4, 260F9-RPE.

additive antitumor activity was observed with each combination of two reagents. With two of the three combinations, additive effects appeared to relate, in part, to the presence of subpopulations that lacked one but not both determinants. Antigen density has been an important determinant of susceptibility to immunotoxins in other systems. Laurent et al. (26) isolated four distinct populations of the CEM T-lymphoblastic cell line which expressed different densities of CD5 molecules. Antitumor activity of T101-RTA conjugates revealed a 3-log difference between cells expressing 500 CD5 molecules and those expressing 40,000 CD5 molecules. In our present study, additive binding was produced by one combination of immunotoxins, and the total concentration of immunoconjugates could be an important determinant of tumor cell killing with these particular reagents. Experiments with the 741F8-rRTA may have been influenced by the intense binding of the antibody to most cells within the SKBr3 line. It is possible that binding of two immunotoxins resulted in a net increase in intracellular conjugate molecules over a threshold limit leading to effective killing. Different immunotoxins might also traffic by different routes, accounting for the log-additive effects observed. Immunocytochemical analysis (27) has shown that antibody-toxin conjugates are internalized by coated vesicles. Once inside the cells, immunotoxin was compartmentalized and the majority of the conjugate was found in the lysosomes (28).

Using immunotoxins in combination, 4–5 log of malignant...
cells could be removed. The clonogenic efficiency of breast cancer target cell lines varied between experiments. Within representative experiments, however, individual immunotoxins could eliminate approximately 2 log of clonogenic tumor cells and combinations approximately 4 log of SKBr3 cells. Whereas log-additive effects were regularly observed with these particular immunotoxins, synergistic interactions were not encountered. Isobolograms fell within the envelope of additivity which had been plotted to compensate for the fact that dose-response curves for the individual immunotoxins were not linear. In earlier studies, investigators have not generally found more complete tumor cell elimination with multiple immunotoxins than with a single reagent. Bregni et al. (5) could produce no greater elimination of Burkitt’s lymphoma cell lines using multiple ricin A chain immunotoxins than they could with a single immunotoxin used at optimal concentration. Stong et al. (29) treated lymphoblastic leukemia cells with a combination of two or four immunotoxins and failed to show any increase in the clonogenic elimination of tumor cells, when compared to the cytotoxicity elicited by one of the most effective conjugates.

The clinical utility of immunotoxins in combination will depend upon detecting additive or synergistic activity against tumor and subadditive activity against critical normal tissues. To the extent that tumor cells coexpress multiple epitopes and that normal cells express only a single epitope, a substantial therapeutic index might be obtained, even when expression of individual antigens is not restricted to neoplastic tissues. One application of the reagents evaluated in the present study is likely to be in the ex vivo elimination of breast cancer cells from human bone marrow, where none of the three immunotoxins appear to bind to normal bone marrow or to destroy the normal hematopoietic colonies that can be measured in cell culture. Here, additive antitumor activity might be anticipated, with little toxicity for normal precursors.

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REFERENCES

Use of Immunotoxins in Combination to Inhibit Clonogenic Growth of Human Breast Carcinoma Cells
